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(54) Title: METHODS AND USES OF CONNECTIVE TISSUE GROWTH FACTOR AS AN INDUCTION AGENT (57) Abstract The present invention relates to novel methods and compositions related to the administration of connective tissue growth factor, alone or in combination with other growth factors, compositions or compounds, to induce the formation of connective tissue, including bone, cartilage, and the skin.		

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**METHODS AND USES OF
CONNECTIVE TISSUE GROWTH FACTOR
AS AN INDUCTION AGENT**

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10 may have certain rights in the invention disclosed in this Specification.

1. STATEMENT OF RELATED CASES

This application is related to and is a continuation-in-
15 part application of Serial Number 08/459,717, entitled "Connective Tissue Growth Factor," filed June 2, 1995, which is a continuation-in-part application of Serial No. 08/386,680, filed on February 10, 1995, having the same title, which is a divisional application of Serial Number
20 08/167,628, filed December 14, 1993, now issued as U.S. Patent No. 5,408,040, which is a continuation of Serial No. 07/752,427, filed on August 30, 1991, now abandoned.

2. FIELD OF THE INVENTION

25 This invention relates generally to the field of growth factors and specifically to Connective Tissue Growth Factor (CTGF) and methods of use thereof.

3. BACKGROUND OF THE INVENTION

30 **A. The Role Of Growth Factors In Bone And Cartilage Formation**

Bone And Cartilage Formation. The formation of tissue and organs in all multicellular organisms that arise
35 from a single fertilized egg requires the differentiation of specialized cell types from non-differentiated stem cells. As embryogenesis proceeds, more highly specialized cell types and complex structures are formed. Currently, however, little concrete information is available on the identification of

the specific factors or the mechanism of action of these factors on skeletal or cartilage formation in vertebrate animals, including humans.

There are two common types of bone formation in the mammalian system: intramembranous ossification and endochondral ossification. The formation of the bones of the skull are an example of intramembranous ossification. There, mesenchymal cells from the neural crest interact with the extracellular matrix of the cranial epithelial cells and form bone. Hall, *Amer. Sci.*, 1988, 76174-181. Mesenchymal cells condense into small islands and differentiate into osteoblasts and capillaries. The osteoblasts secrete a specific type of extracellular matrix, (osteoid) which is capable of binding calcium salts.

Endochondral ossification is the process by which the long bones of the axial skeleton (arms and legs), and the vertebra and ribs form. Hall, *supra*. During this process the formation of bone occurs via a cartilaginous tissue intermediate stage. In mammals, the long bones form from certain mesenchymal cells in the embryonic limb buds. These cells form chondrocytes, and secrete a cartilaginous matrix. Other surrounding mesenchymal cells form the perichondrium (ultimately, the periosteum). In some cases, chondrocytes adjacent to the region where chondrocytes are proliferating and forming differentiate into hypertrophic chondrocytes.

Hypertrophic chondrocytes produce a different type of matrix, and alter their tissue orientation to form the physis. The structure of the physis is arranged in multiple cellular columns composed of zones of cellular hypertrophy, proliferation, ossification and vascularization. Hall, *supra*; Gilbert, "Transcriptional regulation of gene expression," *DEVELOPMENTAL BIOLOGY*, 5th ed. Sinaur Assoc., p. 387-390 (1994). This results in a gradation of cell transformation from chondrocytes to osteoblasts which form the mineralized bone.

Endochondral ossification is an active, ongoing process that occurs in mammals during the growth from infant to adult. The differentiation of mesenchymal cells to

chondrocytes, their proliferation and replacement by osteoblasts are dependent on growth factors (including the TGF- β family), and on the mineralization of the matrix.

5 Tuan, 1984, *J. Exp. Zool.* (suppl.) 1:1-13 (1984); Syfestad and Caplan, 1984, *Devel. Biol.* 104:348-386.

With regard to connective tissue, it is felt that all skeletal elements in mammals are derived from a single stem cell that is capable of differentiating into the specific cell types that compose muscle, cartilage, bone and tendon.
10 These cells also appear to be capable of differentiating into adipose tissue.

The Relevant Art Related To Growth Factors And The Formation Of Bone And Cartilage. Prior to the present invention, it was known generally that growth factors
15 comprise a class of secreted polypeptides that stimulate target cells to proliferate, differentiate and organize developing tissues. Typically, a growth factor's activity is dependent on its ability to bind to specific receptors, thereby stimulating a signaling event within the cell.
20 Examples of some well-studied growth factors include platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I), transforming growth factor beta family (TGF- β), transforming growth factor alpha (TGF- α), epidermal growth factor (EGF), and fibroblast growth factors (FGF).

25 *Effect Of TGF- β On Chondrocyte Growth, Differentiation and Cartilage Formation.* The TGF- β s play a role in chondrogenesis. As previously reported, TGF- β 1 and TGF- β 2 increase chondrogenesis in embryonic rat mesenchymal cells (Seyedin, et al., 1987, *J. Biol. Chem.* 262: 1946-1947),
30 and either isoform can induce formation of chondroblasts from murine muscle mesenchymal cells in culture. Seyedin. et al., 1986, *J. Biol. Chem.* 261: 5693-5695. Application of the TGF- β s to murine embryonic prechondroid tissues increases differentiation of mesenchymal cells, production of
35 proteoglycans, and replication of chondroblasts. Centrella, et al., 1994, *Endocrine Reviews* 15:27-38; Thorp and Jakowlew, 1994, *Bone* 15: 59-64.

Using in-situ hybridization, decreased levels of TGF- β 3 were found in the growth plates of animals with three separate disorders where chondrocytes cease to differentiate. *Id.* In organ cultures of bovine articular cartilage, type II collagen and proteoglycan synthesis were increased after TGF- β administration. Morales and Roberts, 1988, *J. Biol. Chem.* 263: 12828- 12831. In contrast, the TGF- β s have been shown to decrease expression of type II and type X cartilage-specific collagens, synthesis of chondrocyte proteoglycans, and activity of alkaline phosphatase in cultured chondroid cells. Mundy. "The effects of TGF- β on bone," *Clinical Applications of TGF- β .*, 1991, Wiley Chichester, Ciba Foundation Symposium 157: 137-151. Rabbit growth plate chondrocyte differentiation is inhibited by TGF- β , while growth plate chondrocyte mitogenesis is increased. Kato, et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 9552-9556. In addition, large concentrations of TGF- β 1 or TGF- β 2 added to an osteoinductive model favor cartilage, rather than the preference for bone formation, when smaller doses are used. Mundy, supra. This accumulation of apparently contradictory data has hindered efforts to define a function for the TGF- β s in chondrogenesis.

The Bone Morphogenic Proteins And Bone Formation. A family of proteins termed the bone morphogenetic proteins (BMP's are capable of inducing ectopic bone formation in certain mammalian species. With the exception of BMP-1, which encodes a metalloprotease, all of these proteins have structures that are related to TGF- β . However, it is not known which, if any of the BMP's are responsible for the regulation of bone formation during normal embryogenesis.

BMP's were first isolated from demineralized bone as factors that induced bone at extra skeletal ectopic sites. Three peptides were originally identified as BMP-1, BMP-2A, and BMP-3. Celeste, et al., 1990, *Proc. Natl Acad. Sci. USA* 87: 9843-9847; Kubler and Urist, 1990, *Clin. Orthopedics and Rel. Res.* 258: 279-294. The latter two BMPs are members of the TGF- β superfamily. Subsequently, five more closely related members of the BMP group have been identified and

cloned. BMP-5, BMP-6, and BMP-7 are most similar to vgr/60A, while BMP-2 and BMP-4 are more similar to Decapentaplegic. Both vga/60A and Decapentaplegic are Drosophila genes that control dorsal/ventral axis pattern formation. Hoffman,
5 1992, *Mol. Repro and Dev.* 32: 173-178.

In-situ hybridization has localized BMP's gene transcription to areas of bone formation in the limb bud at specific times during development, suggesting a physiologic role. The BMPs induce adventitial post-fetal mesenchymal
10 cells to switch from fibrogenetic to chondroosteoprogenetic patterning. Kubler and Urist, *supra*. Several lines of data suggest the BMPs may act synergistically with TGF- β s to initiate the cascade of osteoinduction in-vivo. In murine subcutis, TGF- β 1 enhances the production of ectopic bone by
15 most BMPs. BMP-6 (also known as VGR-1) is expressed in hypertrophic cartilage at the same time and in the same areas as the TGF- β s, and is associated with collagen type X expression. See, Celeste, et al., *supra*.

The addition of TGF β -2 to bone explants which have been
20 treated with either BMP-2 or BMP-3 results in increased osteoinductive activity and an increased ratio of cartilage to bone when compared to either factor alone. Bentz, et al., 1991, *Matrix* 11:269-275. However, the synergistic effect of these proteins by the TGF- β s is not universal. TGF- β 1 has
25 been shown to directly decrease BMP-2 expression in fetal rat calvaria cultures. Harris, et al., 1994, *J. Bone and Mineral Res.* 9: 855-863. Since BMP-2 is apparently important in bone cell differentiation, it has been suggested that TGF- β 1 may be acting as a switch to monitor the differentiation fates of
30 chondro- or osteo- blastic precursors.

Other Factors Found To Be Expressed In Developing Tissue. Cyr61 is a growth regulator which has been found to be expressed in developing mouse embryo and extraembryonic tissues. O'Brien and Lau, 1992, *Cell Growth Differ.* 3:645-
35 654. Cyr61 is related to but distinct from CTGF and prior to the instant invention, the specific activity of Cyr61 was not known.

B. The Role Of Growth Factors In Wound Healing

Platelet Derived Growth Factor And Wound Healing.

PDGF is a dimeric molecule consisting of an A chain and a B chain. The chains form heterodimers of homodimers and all combinations isolated to date are biologically active. With respect to the factor's activity, PDGF has been characterized as a cationic, heat-stable protein found in the α -granules of circulating platelets. The molecule has been further characterized as a mitogen and a chemotactic agent for connective tissue cells such as fibroblasts and smooth muscle cells.

Because of PDGF's biological activity and release during wound healing, PDGF has been identified as a growth factor involved in wound healing, as well as pathological conditions showing on overproduction of connective tissue, including atherosclerosis and fibrotic diseases.

It has been hypothesized that growth factors other than PDGF may play a role in the normal development, growth, and repair of human tissue.

TGF- β And Wound Healing. The formation of new and regenerating tissue requires the coordinate regulation of various genes that produce both regulatory and structural molecules which participate in the process of cell growth and tissue organization. As with bone induction, it appears that TGF- β plays a central regulatory component of this process. TGF- β is released by platelets, macrophages and neutrophils which are present in the initial phases of the repair process. TGF- β can act as a growth stimulatory factor for mesenchymal cells and as a growth inhibitory factor for endothelial and epithelial cells. It has been suggested that the growth stimulatory action of TGF- β appears to be mediated via an indirect mechanism involving the induction of other genes including growth factors such as PDGF.

Several members of the TGF- β superfamily possess activities suggesting possible applications for the treatment of cell proliferative disorders, such as cancer. In particular, TGF- β has been shown to be potent growth inhibitor for a variety of cell types (Massague, 1987, Cell

49:437), MIS has been shown to inhibit the growth of human endometrial carcinoma tumors in nude mice (Donahoe, et al., 1981, *Ann. Surg.* 194:472), and inhibition has been shown to suppress the development of tumors both in the ovary and in the testis (Matzuk, et al., 1992, *Nature*, 360:313).

Many of the members of the TGF- β family are also important mediators of tissue repair. TGF- β has been shown to have marked effects on the formation of collagen and causes of striking angiogenic response in the newborn mouse (Roberts, et al., 1986, *Proc. Natl. Acad. Sci., USA* 83:4167). The bone morphogenic proteins (BMPs) can induce new bone growth and are effective for the treatment of fractures and other skeletal defects (Glowacki, et al., 1981 *Lancet*, 1:959; Ferguson, et al., 1988, *Clin. Orthoped. Relat. Res.*, 227:265; Johnson, et al., 1988, *Clin. Orthoped. Relat. Res.*, 230:257).

C. Connective Tissue Growth Factor

A previously unknown growth factor, related to PDGF, and termed Connective Tissue Growth Factor (CTGF), has been reported in a related patent. See, U.S. Patent No. 5,408,040. CTGF is a cysteine-rich mitogenic peptide which is selectively induced in fibroblasts after activation with TGF- β . Igarashi, et al., 1993, *Mol. Biol. Cell* 4:637-645.

CTGF is a member of a family of peptides that include serum induced gene products cef10 (Simmons, et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:1178-1182), cyr61 (O'Brien, et al., 1990, *Mol. Cell. Biol.* 10:3569-3577), fisp12/IG M1 (Ryseck, et al., 1993, *Cell Growth & Differ.* 2:225-233), and a chicken transforming gene, nov (Joliot, et al. 1992, *Mol. Cell Biol.* 12:10-21 (1992)). CTGF also shares sequence homology with a drosophila gene product, twisted gastrulation (twg) (Mason, et al., 1994, *Genes & Develop.* 8:1489-1501), which determines cell fates during dorsal/ventral pattern formation in the embryo.

As reported in that patent, CTGF is the product of a distinct gene. As also reported in U.S. Patent No. 5,408,040, CTGF possesses mitogenic activity. The ultimate result of this mitogenic activity in vivo, is the growth of

targeted tissue. CTGF also possesses chemotactic activity, which is the chemically induced movement of cells as a result of interaction with particular molecules.

Although the molecule is antigenically related to PDGF, there is little if any peptide sequence homology between CTGF and PDGF. Anti-PDGF antibody has high affinity to the non-reduced forms of the PDGF isomers and the CTGF molecule and ten-fold less affinity to the reduced forms of these peptides, which lack biological activity.

A second protein, identified as "connective tissue growth factor-2" or "CTGF-2," has been reported also. See, PCT Application No. PCT/US94/07736 (International Publication No. WO 96/01896). According to the PCT Application, CTGF-2 may also be used to enhance the repair of connective and support tissue. Although identified as a connective tissue growth factor, CTGF-2 is not closely related to the CTGF of the present invention. Specifically, the CTGF family is comprised of three distinct groups of proteins: CTGF/Fisp12, cyr61 and nov. The protein of the claimed invention falls within the first group of proteins, as compared to CTGF-2, which falls with the cyr61 group. PCT Application No. PCT/US94/07736 at 4.

Notwithstanding the identification of various PDGF related growth factors, including CTGF, prior to the present invention, such factors have not been proven to be an effective induction agent for the production of matrices, including the induction of bone and/or cartilage tissue.

4. SUMMARY OF THE INVENTION

The subject invention provides novel methods and compositions for the treatment of diseases, disorders or ailments wherein matrix and/or connective tissue production, including the production of bone and/or cartilage, is desired. The subject invention is likewise directed to the treatment of diseases, disorders or ailments wherein the promotion of wound healing is desired.

More specifically, the compositions of the present invention comprise CTGF and/or fragments and/or derivatives

thereof (hereinafter collectively "CTGF"), alone or in combination with other growth factors. The CTGF used in the subject compositions may be either obtained by isolation from natural sources, synthetic manufacture, or production by recombinant genetic engineering techniques.

In one aspect of the invention, the methods of the present invention comprise the administration of an effective amount of CTGF, alone or in combination with one or more compounds, to treat diseases, disorders or ailments wherein the induction of bone or cartilage tissue is desired. In a preferred embodiment of this method, such additional compound is a growth factor.

In another aspect of the invention, the methods of the present invention comprise the administration of an effective amount of CTGF, alone or in combination with one or more compounds, again preferably one or more growth factors, to treat diseases, disorders or ailments wherein the promotion of wound healing is desired.

In a preferred embodiment of the invention, the composition comprising CTGF is administered directly onto or into the site in which bone or cartilage induction is desired so as to induce the formation of such bone or cartilage. In another embodiment, the composition is formulated for targeted delivery or alternatively, are designed for the release of the novel compositions in the relevant site (e.g., the wound in which cartilage formation is desired). In each case, the CTGF containing composition is appropriately formulated for administration to a patient in need.

5. DEFINITIONS

As used in this Specification, the term "CTGF" shall mean: (1) a protein encoded by the amino acid sequence set forth at Figure 1C, (2) a protein having CTGF activity wherein such protein is encoded by the amino acid sequence of Figure 1C wherein one or more amino acids have been added, deleted, mutated, substituted or otherwise altered ("derivative") and the nucleotide sequence encoding said protein can hybridize to the nucleic acid sequence of Figure

1C under stringent conditions, or (3) a fragment of CTGF or a derivative thereof.

As used in this Specification, the term "induce," as used herein, shall mean to produce, form, to cause to produce, or to cause to form.

As used in this Specification, the phrase "induction agent" shall mean an agent, including proteins or other biological materials, which causes the production or formation of a specific end result (e.g., the production of connective tissue).

As used in this Specification, the term "polynucleotide" denotes DNA, cDNA and/or RNA which encode untranslated sequences which flank the structural gene encoding CTGF. For example, a polynucleotide of the invention includes 5' regulatory nucleotide sequences and 3' untranslated sequences associated with the CTGF structural gene. A polynucleotide of the invention which includes the 5' and 3' untranslated region is illustrated in FIGURE 1C. The 5' regulatory region, including the promoter, is illustrated in FIGURE 1B. A more detailed description of the polynucleotides contemplated by the present invention may be found at U.S. Patent No. 5,408,040.

As used in this Specification, the phrase "stringent conditions," as used herein, refers to those hybridizing conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

As used in this Specification, the phrase "recombinant expression vector" refers to a plasmid, virus or other

vehicle known in the art that has been manipulated by insertion or incorporation of the CTGF genetic sequences.

As used in this Specification, the phrase "therapeutically effective" means that amount of CTGF which is effective in inducing bone or cartilage formation or wound healing.

6. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A shows the structural organization of the CTGF gene. Exons are indicated by boxed regions, with solid areas in the gene corresponding to the open reading frame.

FIGURE 1B shows a comparison of nucleotide sequences between CTGF promoter and fisp-12 promoter. Identical nucleotides are marked with asterisks. The TATA box and other consensus sequences are indicated and shaded. The site of transcriptional initiation is indicated at position number +1.

FIGURE 1C shows the complete nucleotide and deduced amino acid sequence for the CTGF structural gene and 5' and 3' untranslated sequences.

FIGURE 2 shows experimental *in situ* hybridization results related to the expression of CTGF transcripts in the growth plate of long bones in newborn mice. The *in situ* hybridization experiments were performed using an anti-sense CTGF RNA probe as described below. Chondrocytes in the proliferation zone are strongly positive for CTGF gene expression, indicating that CTGF is produced at sites of cartilage growth.

FIGURE 3 shows the expression of the CTGF gene during embryogenesis wherein a transgenic mouse is constructed using a fusion gene constructed from a CTGF promoter and a β -galactosidase structural gene. This gene, introduced into the germ line, expresses β -galactosidase at sites of CTGF expression and can be detected by histochemical means by expressing sections of the developing transgenic animal to the substrate X-gal which deposits a blue color at sites of β -galactosidase activity. Panel A is a 12 day mouse embryo from such a transgenic mouse. The blue staining is an area

destined to become Meckel's cartilage, which is the first cartilage to form. Panel B is a photograph of the hind limb and paw which demonstrates staining at the ends of the long bones and in the paw in the growth plates of the metatarsal.

5 FIGURE 4 provides evidence of induction of cartilage and bone in cultures of C3H10T1/2 mouse embryonic stem cells. C3H10T1/2 cells were cultured as described under methods. Cells were treated with either nothing (Panel A), 5 -
10 azacytodine (Panel B), CTGF at 50 ng/ml (Panel C) or 5-azacytodine followed by CTGF (Panel D).

FIGURE 5 sets forth Northern blot analysis of CTGF gene expression in wound chambers implanted at sites of bone regeneration.

15 FIGURE 6 sets forth evidence related to the expression of CTGF in human osteoblasts in response to TGF- β .

FIGURES 7A-7D set forth results of a chondrogenic assay.

FIGURE 7A provides the chondrogenic assay results for the control culture.

20 FIGURE 7B provides the chondrogenic assay results for a culture in which 5 ng/ml TGF- β 1 was added.

FIGURE 7C provides the chondrogenic assay results for a culture in which 5 ng/ml TGF- β 1 and 10 ng cholera toxin were added.

25 FIGURE 7D provides the chondrogenic assay results for a culture in which 5 ng/ml TGF- β 1, 10 ng/ml cholera toxin, and 5 ng/ml CTGF were added.

FIGURE 8A is a Scatchard Plot reflecting CTGF binding to NRK cells.

30 FIGURE 8B is a Scatchard Plot reflecting CTGF binding to rat chondroblasts.

7. DETAILED DESCRIPTION OF THE INVENTION

7.1. Methods For Making CTGF

35 *Nucleic Acid Sequences Encoding CTGF.* In accordance with the invention, nucleotide sequences encoding CTGF or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of the protein or a functional equivalent thereof, in

appropriate host cells. Alternatively, nucleotide sequences which hybridize, under stringent position, to portions of the CTGF sequence may also be used in nucleic acid hybridization assays, Southern and Northern blot analyses, etc. In yet
5 another method, DNA molecules encoding CTGF may be isolated by hybridization procedures comprising antibody screening of expression libraries to detect shared structural features.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a
10 functionally equivalent amino acid sequence, may be isolated and used in the practice of the invention for the cloning and expression of CTGF. Such DNA sequences include those which are capable of hybridizing to the human CTGF sequence under stringent conditions.

15 Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions,
20 additions or substitutions of amino acid residues within the CTGF sequence, which result in a silent change thus producing a functionally equivalent protein. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity,
25 and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the
30 following: leucine, isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

The DNA sequences of the invention may be engineered in order to alter the protein's sequence for a variety of ends
35 including but not limited to alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis to, for

example, insert new restriction sites. For example, in certain expression systems such as yeast, host cells may over- glycosylate the gene product. When using such expression systems it may be preferable to alter CTGF coding
5 sequence to eliminate any N-linked glycosylation site.

The CTGF sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric CTGF protein expressing a heterologous epitope that
10 is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the CTGF sequence and the heterologous protein sequence (e.g. a sequence encoding a growth factor related to PDGF), so that CTGF can be cleaved away from the
15 heterologous moiety.

The coding sequence of CTGF may also be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, *Nucleic Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 1980, *Nucleic Acids Res.* 9(10):2331; Matteucci and Caruthers, 1980, *Tetrahedron Letters* 21:719; and Chow and Kempe, 1981, *Nucleic Acids Res.* 9(12):2807-2817. Alternatively, the protein
20 itself could be produced using chemical methods to synthesize the CTGF amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. See e.g., Creighton, 1983, *Proteins Structures And Molecular Principles*, W.H. Freeman and Co., N.Y. pp. 50-60. The
25 composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing. See e.g., for the Edman degradation procedure, see, Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 34-49.

35 A more detailed description of the nucleic acid sequences comprising the present invention and methods for identifying such sequences may be found in U.S. Patent,

Serial No. 5,408,040, which is incorporated herein by reference.

Expression Of CTGF. In order to express a biologically active CTGF, the nucleotide sequence coding for the protein, or a functional equivalent as described above, *supra*, was inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

More specifically, methods which are well known to those skilled in the art can be used to construct expression vectors containing the CTGF sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See e.g., the techniques described in Maniatis et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the CTGF coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the CTGF coding sequence; yeast, including *Pichia pastoris* and *Hansenula polymorpha*, transformed with recombinant expression vectors containing the CTGF coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the CTGF coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the CTGF coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus, human tumor cells (including HT-1080)) including cell lines engineered to contain multiple copies of the CTGF DNA either

stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines). As used herein, it is understood that the term "host-expression vector systems" and more generally, the term "host cells" includes any progeny of the host cell or host-expression vector system. It is further understood that although all progeny may not be identical to the parental cell, as mutations may occur during replication, such progeny are included in the scope of the invention.

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the CTGF DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the expressed CTGF. For example, a suitable vector for expression in bacteria includes the T7-based vector as described in Rosenberg, et al., 1987, Gene 56:125. As further example, when large quantities of CTGF are to be

produced to screen peptide libraries, vectors which direct the expression of high levels of protein products that are readily purified may be desirable. Such vectors include but are not limited to the *E. coli* expression vector pUR278 (Ruther et al., 1983, *EMBO J.* 2:1791), in which the CTGF coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke & Schuster, 1989, *J. Biol. Chem.* 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides such as CTGF with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

More generally, where the host is a procarote, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth and subsequently treated by the CaCl_2 , or alternatively MgCl_2 or RbCl , method using procedures well known in the art.

Where the host cell is a eucaryote, various methods of DNA transfer can be used. These include transfection of DNA by calcium phosphate-precipitates, conventional mechanical procedures, including microinjection, insertion of a plasmid encased in liposomes, or use of virus vectors. Eucaryotic cells may also be cotransformed with DNA sequences encoding the polypeptide of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eucaryotic cells and express protein. See, *Eukaryotic Viral Vectors*, 1992, Cold Spring Harbor Laboratory, Gluzman, Ed.). Eucaryotic host cells include yeast, mammalian cells, insect cells and plant cells.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, *Current Protocols in Molecular Biology*, Vol. 2, 1988, Ausubel et al., Ed., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, *Methods in Enzymology*, Wu & Grossman, Eds., Acad. Press, N.Y., 153:516-544; Glover, 1986, *DNA Cloning*, Vol. II, IRL Press, Wash., D.C., Ch. 3; Bitter, 1987, *Heterologous Gene Expression in Yeast*, *Methods in Enzymology*, Berger & Kimmel, Eds., Acad. Press, N.Y., 152:673-684; and *The Molecular Biology of the Yeast Saccharomyces*, 1982, Strathern et al., Eds., Cold Spring Harbor Press, Vols. I and II. For example, various shuttle vectors for the expression of foreign genes in yeast have been reported. Heinemann, et al., 1989, *Nature* 340:205; Rose, et al., 1987, *Gene* 60:237.

In cases where plant expression vectors are used, the expression of the CTGF coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, *Nature* 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, *EMBO J.* 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, *EMBO J.* 3:1671-1680; Broglie et al., 1984, *Science* 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, *Mol. Cell. Biol.* 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques, see, e.g., Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp. 421-463; Grierson & Corey, 1988, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9.

In an insect system, an alternative expression system could be used to express CTGF. In one such system, Baculovirus is used as a vector to express foreign genes. The virus then grows in the insect cells. The CTGF coding sequence may be cloned into non-essential regions (for

example the polyhedrin gene) of the virus and placed under control of a Baculovirus promoter. These recombinant viruses are then used to infect insect cells in which the inserted gene is expressed. See, e.g., Smith et al., 1983, 5 J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051.

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the CTGF coding sequence may be ligated to an adenovirus 10 transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a 15 recombinant virus that is viable and capable of expressing CTGF in infected hosts. See e.g., Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659. Alternatively, the vaccinia 7.5K promoter may be used. See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett 20 et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931.

In another embodiment, the CTGF sequence is expressed in human tumor cells, such as HT-1080, which have been stably transfected with calcium phosphate precipitation and a 25 neomycin resistance gene. In yet another embodiment, the pMSXND expression vector or the like is used for expression in a variety of mammalian cells, including COS, BHK 293 and CHO cells. Lee and Nathans, 1988, J. Biol. Chem. 263:3521.

Specific initiation signals may also be required for 30 efficient translation of inserted CTGF coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire CTGF gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional 35 translational control signals may be needed. However, in cases where only a portion of the CTGF coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the

into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

5 A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. (USA)* 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, *Cell* 22:817) genes can be employed in tk⁻, hgp⁺ or apt⁻ cells,
10 respectively.

Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, *Proc. Natl. Acad. Sci. (USA)* 77:3567; O'Hare, et al., 1981, *Proc. Natl. Acad. Sci. (USA)* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. (USA)* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, *J. Mol. Biol.* 150:1); and hyg⁺, which confers resistance to hygromycin (Santerre, et al., 1984, *Gene* 30:147) genes. Recently, additional
20 selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, *Proc. Natl. Acad. Sci. (USA)* 85:8047), and ODC (ornithine decarboxylase) which confers
25 resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory).

30 The isolation and purification of host cell expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibody.

35

acids of the host cell may be extracted and assayed for hybridization to such probes.

5 The fourth approach involves the detection of the biologically active or immunologically reactive CTGF gene product. A number of assays can be used to detect CTGF activity including but not limited to those assays described in U.S. Patent No. 5,408,040.

7.3. Treatment Indications

10 The methods, compounds and formulations of the present invention are each directed to the treatment of disorders, diseases or ailments related to the underproduction of connective tissue in bone, cartilage, or other organs such as skin and muscle alternatively, to
15 disorders, diseases or ailments in which the formation of bone or cartilage is desired.

These diseases, disorders or ailments include the repair of cartilage or bone defect after a variety of traumatic injuries or disorders including arthritis, osteoporosis and
20 other skeletal disorders, hypertrophic scars, burns, and vascular hypertrophy. Because these problems are due to a poor growth response of the fibroblasts, stem cells, chondrocytes, osteoblasts or fibroblasts at the site of injury, the addition of a biologically active agent which
25 could stimulate the growth of these cells would be beneficial.

Another important use of CTGF would be in culture systems to expand stem cells or chondrocytes that were removed from an individual prior to reimplantation. In a
30 similar process, CTGF could be added to either stem cells or chondrocytes when they were to be added as a graft to help stimulate the expansion and differentiation of these cells at the site of implantation. CTGF could also be added to a graft composed of cartilage or bone to help stimulate growth.

35 Another treatment indication is directed to administering CTGF to a patient in need to enhance wound healing. PDGF and other growth factors, such as CTGF, are involved in normal healing of skin wounds. The CTGF

part, by promoting the growth of connective tissue. In another embodiment, CTGF may be administered in combination with proteins or compounds which are believed to promote the formation of connective tissue.

5 Whether the composition is comprised of CTGF alone or CTGF and additional agents as the active ingredient, such composition is prepared by combining, in a pharmaceutically acceptable carrier substance, e.g., inert gels or liquids, the purified CTGF and TGF- β .

10 A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co.,
15 Easton, PA, latest edition.

7.4.1. Routes Of Administration.

 Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal
20 administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

 Alternately, one may administer the compound in a local
25 rather than systemic manner, for example, via injection of the compound directly into an area requiring CTGF, often in a depot or sustained release formulation.

 Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with
30 a specific antibody, targeting, for example, cartilage. The liposomes will be targeted to and taken up selectively by the afflicted tissue.

7.4.2. Composition/Formulation.

35 The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving,

may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The molecules may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-

alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic molecules may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars,

circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of
5 administration and dosage can be chosen by the individual physician in view of the patient's condition. See, e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1.

Dosage amount and interval may be adjusted individually
10 to provide plasma levels of the active moiety which are sufficient to maintain the induction effects of CTGF, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data; for example, the concentration necessary to achieve 50-90%
15 activity of CTGF to induce bone growth using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

20 Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

25 In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of
30 administration and the judgment of the prescribing physician.

7.4.4. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit
35 dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions

8. EXAMPLES

The connective tissue growth factor gene is not only expressed in fibroblasts but is selectively induced by TGF- β only in mesenchymally derived connective tissue cells (i.e., fibroblasts, smooth muscle, chondrocytes, osteoblasts, astroglial cells, etc.). The expression of the gene connective tissue cells that form the skeletal elements in vertebrates indicate that CTGF plays a role in the formation of cartilage, bone tendon and muscle in the vertebrate animal. The results of the following examples demonstrate that CTGF can regulate the induction, differentiation and growth of cells which form both cartilage and bone in vertebrate animals, including humans. Specifically, the results provide that: (1) CTGF transcripts are present in the growth plate of long bones in adult rats and newborn mice; (2) The CTGF gene is expressed at sites of cartilage induction and growth in embryonic mice; (3) CTGF receptors are present on rat chondrocytes; (4) The CTGF gene is expressed at site of bone regeneration after injury in adult rabbits; (5) The CTGF protein can induce pluripotent mouse embryonic stem cell lines to differentiate in to chondrocytes and osteoblasts; (6) Human osteoblasts produce CTGF in culture.

8.1 Biological Assays

Methods: Mitogenic And Anchorage Independent Growth Assays. Mitogenic assays were performed in monolayer cultures using 48 well plates and NRK fibroblasts as target cells as described previously in Grotendorst, et al. 1991, *J. Cell Physiol.* 149:235-243. Anchorage independent growth assays were performed essentially as described in Guadagno and Assoian, 1991, *J. Cell Biol.* 115:1572-1575.

Methods: Extracellular Matrix Protein mRNA Induction Assays. NRK rat fibroblasts were grown to confluence in Dulbecco's modified eagle media with 5% fetal bovine serum and then serum starved in DMEM with 1% bovine serum albumin for 24 hours. Growth factors were added to the cell cultures and total cellular RNA was extracted after 24

20 μ g/ml proteinase K in 50 mM Tris-HCl pH 7.4, 5 mM EDTA, refixed in 4.0% paraformaldehyde and dipped in 0.1 M triethanolamine and 1 ml acetic anhydride, prior to dehydration in sequentially graded alcohols. Both sense and antisense CTGF RNA probes were constructed using a riboprobe kit (Promega, Madison, WI) with T7 and Sp6 promoters, respectively. The specific activity of the probes was 1 x 10⁸ cpm/ μ g RNA. Slides were hybridized overnight in 50% deionized formamide, 10% dextran sulfate, 50 mM DTT, 0.3M NaCl, 0.01 M Tris pH 7.5, 5 mM EDTA, 10 mM Na₂HPO₄, 0.02 % Ficoll, 0.02 % PVP, 0.02 % BSA, 0.2 mg/ml yeast tRNA and the riboprobe (5 x 10⁴ cpm/ μ l) under a coverslip at 54° C. Slides were washed in 250 ml 5X SSC, 10 mM beta mercaptoethanol at 50° C for 30 minutes, 2x SSC, 100mM beta mercaptoethanol, 50% formamide at 65° C for 20 minutes and 3 times in TEN buffer (1 M Tris, 0.5 M EDTA, 5M NaCl) for 10 minutes. The second TEN wash included 10 μ g RNase A. The final two washes were in 2X SSC at 65° C. for 15 minutes each. After dehydrating again through graded alcohols with 0.3 M ammonium acetate, the slides were dipped in photographic emulsion (Ilford K-5, Polyscience) and incubated for 8 days at 4° C. Slides were then developed and sections counter stained in Mayer's hematoxylin and eosin.

Results. The results of these studies indicate that the CTGF gene is expressed in the proliferation zone of the growth plate. This zone contains the chondrocytes that are actively replicating to increase the length of the bone. The expression of CTGF at this site is consistent with it functioning as a growth factor for the chondrocytes.

8.3 CTGF Gene Expression At Site Of Cartilage Induction And Growth In Embryonic Mice.

In order to confirm CTGFs role in cartilage induction and growth, the expression of the CTGF gene in mouse embryo's at sites where cartilage and bone will form but have not formed yet was studied. For purposes of this study, a transgenic mouse line which contains a transgene composed of the human CTGF promoter elements which are

Equilibrium Assay. Equilibrium binding assays were performed on confluent monolayers of NRK-49F rat fibroblasts and primary rat articular chondroblasts to determine the number and affinity of CTGF receptors on these cells.

5 Binding was performed in the cold for 4 hours with varying concentrations of iodinated recombinant human CTGF (rhCTGF). Non-specific binding was determined by including a 200-fold molar excess of unlabeled ligand. Representative Scatchard plots are set forth at FIGURE 8A (with respect to equilibrium
10 binding assays performed using NRK cells) and FIGURE 8B (with respect to equilibrium binding assays performed using rat chondroblasts).

Competition Assay. Several cell types were tested for the expression of CTGF receptors including, normal rat
15 kidney fibroblasts, mouse fibroblasts, mink lung epithelial cells and rat articular chondrocytes. CTGF was labeled by iodination with ^{125}I and the radiolabeled CTGF used in competition binding assays to measure CTGF receptors on the various cell types. As set forth in Table 1, below, only the
20 NRK fibroblasts and rat articular chondrocytes expressed high affinity receptors for CTGF. Mouse fibroblasts had few if any high affinity receptors and no binding was detected in the mink lung epithelial cells.

25
TABLE 1
BINDING CHARACTERISTIC FOR rhCTGF ON VARIOUS CELLS

30	Cell Type	<u>High Affinity</u>		<u>Low Affinity</u>	
		KD (pM)	Sites/cell	KD (nM)	Sites/cell
	NRK	13-23	2200-3500	1.1-2.2	126,000-195,000
	Chondrocytes	21	3500-4800	1.0	150,000
35	NIH3T3	5-10	480	1.8	102,000
	MLEC	none detected		none detected	

40 These data indicate that chondrocytes express both CTGF and its receptor and would be therefore capable of responding to CTGF as a growth stimulatory factor.

Second, while some cartilaginous nodules were present most of the nodules appeared to be osteoid (bone). Thus, CTGF can induce the formation of both chondrocytes and osteoblasts from undifferentiated mesenchymal cells. These results demonstrate that this factor could be used to stimulate the differentiation of cartilage and bone where desired.

8.6 CTGF Gene Expression At Site Of Bone Regeneration After Injury In Adult Rabbits.

An experimental model was developed to examine the expression of various regulatory and matrix protein genes during wound repair. In this model, mesh nylon cylinders are implanted in the ilium of the pelvis of male New Zealand white rabbits (10 kg) which had been anesthetized by ether. A 1.1 cm diameter hole was bored in the ilium of the pelvis using a bone trephine and the chamber press fit into the hole. The chamber was anchored in place using flaps of the adjacent musculature and ligaments. Two chambers were implanted in each of twenty animals.

Animals were sacrificed on Days 9, 14, 21, 24, 28, 31, 35, 42 and 56 after implantation of the chambers. The chambers were removed and the tissue on the outside of the chambers were carefully and completely removed. The chambers were then cut open and tissue contained within the chambers was collected.

Total RNA was extracted from the tissue obtained from 6-18 chambers (pooled from 1-3 animals) by Guanidine-isothiocyanate extraction (Chomcaynski and Sacchi, 1987, *Anal. Biochem.* 162:156-159) and CsCl centrifugation (Chirgwin, et al., 1979, *Biochemistry* 18:5294-5299). The amount of RNA recovered ranged from 100-300 μ g during the different days of collection. Total RNA was electrophoresed on an agarose/formaldehyde gel and transferred to nitrocellulose. Equivalent amounts of RNA were transferred as judged from staining of the ribosomal RNA present in each sample on the nitrocellulose filter. The CTGF probe was a 900 base pair fragment which represented the open reading frame of the CTGF cDNA. Hybridizations were performed using 1x10⁶ cpm/ml of

% nonfat powdered milk (TBS-milk), prior to overnight exposure to 2 g/ml chicken anti-human CTGF IgY diluted in TBS-milk. Filters were washed five times in TBS-milk, 5 minutes each, and incubated with alkaline-phosphatase-conjugated affinity purified rabbit anti-chicken IgY (1:1,000 dilution, Organon Teknika-Cappel, West Chester, Pa.) in TBS-milk for 90 minutes. The filters were washed three times with TBS-milk followed by two washes in TBS, and the antigens were detected using a commercial alkaline phosphatase substrate kit (Sigma, St. Louis, MO).

Results. Human osteoblasts were obtained from donors after surgical removal of bone during procedures to remove bone tumors or joint replacement. Osteoblasts were cultured from the bone and identified using standard. Cells were grown to confluence in complete media containing 10 % fetal calf serum and made quiescent by changing the media to serum free media overnight. Some cultures were treated with TGF- β and compared to non-treated cultures. The osteoblasts that were treated with TGF- β were stimulated to produce CTGF, as detected with a specific anti-CTGF antibody. As set forth at FIGURE 5, the media was collected and analyzed for CTGF production and secretion by immunopurification of the CTGF with a CTGF specific antibody and detection and quantitation by western blots using the same antibody. As observed with fibroblasts, smooth muscle cells, and chondrocytes, TGF- β induces CTGF production by the human osteoblasts. Control non-treated cells did not synthesize detectable amounts of CTGF.

As evidenced by this experiment, osteoblasts respond to TGF- β similarly to other connective tissue cells with regard to CTGF production.

8.8 Transgenic Rabbit Models

All mice studies were conducted in accordance with the principles and procedures outlined in "Guidelines for Care and Use of Experimental Animals". Generation of transgenic models was carried out at the University of Miami Transgenic Mouse Core Facility using standard techniques.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

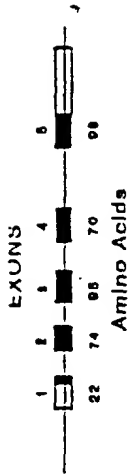
Biological Deposits

The sequence of the CTGF of the invention was deposited with Genbank, Los Alamos National Laboratory, Los Alamos, New Mexico, 87545, USA, on July 26, 1990, and given an accession no. M36965. The deposit of this CTGF sequence is for exemplary purposes only, and should not be taken as an admission by the Applicant that such deposit is necessary for enablement of the claimed subject matter.

In respect of all designated States in which such action is possible and to the extent that it is legally permissible under the law of the designated State, it is requested that a sample of the deposited micro-organism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g., EPC rule 28(4), United Kingdom Patent Rules 1982 rule 17(3), Australian Regulation 3.25(3) and generally similar provisions *mutatis mutandis* for any other designated State.

WHAT IS CLAIMED:

1. A pharmaceutical composition comprising CTGF.
- 5 2. A method for inducing bone formation comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.
- 10 3. The method of claim 2 wherein said composition further comprises a second growth factor.
4. The method of claim 3 wherein the second growth factor is TGF- β .
- 15 5. The method of claim 2 wherein said composition is further comprised of at least one collagen.
6. The method of claim 2 wherein the patient is suffering from an affliction which affects bone formation.
- 20 7. The method of claim 6 wherein the affliction is selected from the group consisting of osteoporosis, osteoarthritis and osteochondritis.
- 25 8. A method for inducing tissue formation comprising the administration to a patient in need a composition comprising CTGF and a pharmaceutical acceptable carrier.
- 30 9. The method of claim 8 wherein said composition further comprises a second growth factor.
10. The method of claim 8 wherein the second growth factor is TGF- β .
- 35 11. The method of claim 8 wherein said composition is further comprised of at least one collagen.

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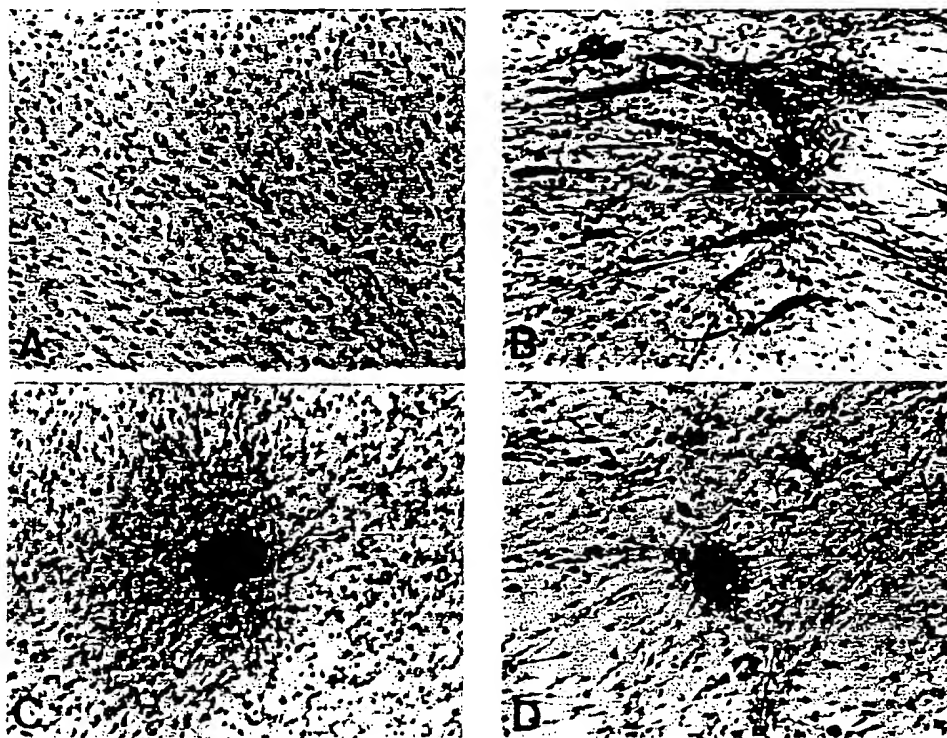
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677 AAACCGCTCGGACTGAGTTTCTTTCTCCAGCTGCTGCCAGCCCCCCCCCTGCAGCCCAGA
737 TCCCAACTCGCATCCCTGACGCTCTGGATGTGAGAGTGCCCCAATGCCTGACCTCTGCAT
797 CCCCCACCCCTCTCTTCCCTTCTTCTCCAGCCAAAGATGGTGCTCCCTGCATCTTCG
..... A K D G A P C I F
857 GTGGTACGGTGTACCGCAGCGGAGAGTCTTCCAGAGCAGCTGCAAGTACCAGTGCACGT
G G T V Y R S G E S F Q S S C K Y Q C T
917 GCCTGGACGGGGCGGTGGGCTGCATGCCCTGTGCAGCATGGACGTTCTGTCTGCCAGCC
C L D G A V G C M P L C S M D V R L P S
977 CTGACTGCCCCCTTCCCGAGGAGGGTCAAGCTGCCCCGGAATGCTGCGAGGAGTGGGTGT
P D C P F P R R V K L P G K C C E E W V
1037 GTGACGAGCCCCAAGGACCAACCGTGGTTGGGCCTGCCCTCGCGGGTGAGTCCAGTCTTC
C D E P K D Q T V V G P A L A
1097 CTCTAAGTCAGGGTCTGTGATTCTCTCCAGGGAGGGAGTCCCTAAGTGTGCCGACCGAACG
1157 GGGAAATACCTTATCAGGCGTTTACATGGTGTGTGTGCTCTGCTCTCGCAGCTTACC
.....Intron 3 A Y
1217 GACTGGAAGACACGTTTGGCCCCAGACCCAACTATGATTAGAGCCAACTGCCTGGTCCAGA
R L E D T F G P D P T M I R A N C L V Q
1277 CCACAGAGTGGAGCGCCTGTTCCAAGACCTGTGGGATGGGCATCTCCACCCGGGTACCA
T T E W S A C S K T C G M G I S T R V T
1337 ATGACAACGCCTCCTGCAGGCTAGAGAAGCAGAGCCGCCTGTGCATGGTTCAGGCCTTGC
N D N A S C R L E K Q S R L C M V R P C
1397 AAGCTGACCTGGAAGAGAACATTAAGGTACATGTTCTGCTCCTATTAATATTTTTTCA
E A D L E E N I KIntron 4
1457 GGAAAAACAGTGGATAGGACCCAACTTAGGGCTCTTGACGCTTGTAGTATAAGCCCGT
1517 TATCTCCAAAACCTATCTAACCATTGAGCTGTTTTGCTGGAATGAGAGCTTGTGTAATAGC
1577 AACCACCAGTTTTTCCAATAAGAAATCTTCCACAGGGTTAGTTAATTCAAGACATTTCAAG
1637 AGAGGCTCTGGCTATTTTTGGACATAGCAAATGAGACTCAAACCTCCTCCCCTCAAAATA
1697 TAAACAGAAGTCAGACAACAGAAGACTAAACACAGAGGGTTGAAGAAAGCCACTCCTCT
1757 TGTAGAGTCGCTGATTTTTTTTTTCTCTCTCTTTCCCTTGTCTTCTTAGAAGGGCA
..... K G
1817 AAAAGTGCATCCGTACTCCCAAATCTCCAAGCCTATCAAGTTTGAGCTTTCTGGCTGCA
K K C I R T P K I S K P I K F E L S G C
1877 CCAGCATGAAGACATACCGAGCTAAATTCTGTGGAGTATGTACCGACGGCCGATCTGCA
T S M K T Y R A K F C G V C T D G R C C
1937 CCCCCACAGAACCACCACCCTGCCGGTGGAGTTCAGTGCCTGACGGCGAGGTCTGA
T P H R T T T L P V E F K C P D G E V M
1997 AGAAGAACATGATGTTTCATCAAGACCTGTGCCTGCCATTACAAGTGTCCCGGAGACAATG
K K N M M F I K T C A C H Y N C P G D N
2057 ACATCTTTGAATCGCTGTACTACAGGAAGATGTACGGAGACATGGCATGAAGCCAGAGAG
D I F E S L Y Y R K M Y G D M A *
2117 TGAGAGACATTAACCTCATTAGACTGGAACCTGAACTGATTACATCTCATTTTTCCGTAA
2177 AAATGATTTTCACTAGCACAAGTTATTTAAATCTGTTTTTCTAACTGGGGGAAAAGATTCC
2237 CACCCAATTCAAAACATTGTGCCATGTCAAACAAATAGTCTATCTTCCCCAGACACTGGT
2297 TTGAAGAATGTAAAGACTTGACAGTGGAACTACATTAGTACACAGCACCAGAATGTATAT
2357 TAAGGTGTGGCTTTAGGAGCAGTGGGAGGTTACCGGCCGGTTAGTATCATCAGATCGAC
2417 TCTTATACGAGTAATATGCTGCTGCTATTGGAAGTGAATTGAGAAGGAAAATTTTAGCGTG
2477 CTCACTGACCTGCCTGTAGCCCCAGTGACAGCTAGGATGTGCATTCTCCAGCCATCAAGA
2537 GACTGAGTCAAGTTGTTCCCTTAAGTCAGAACAGCAGACTCAGCTCTGACATTCTGATTCTG
2597 AATGACACTGTTTCAAGGAATCGGAATCCTGTGCTGATTAGACTGGACAGCTTGTGGCAAGTGA
2657 ATTTGCCTGTAACAAGCCAGATTTTTTAAATTTATATTGTAAATATTGTGTGTGTGTGT
2717 GTGTGTGTATATATATATATATATATGTACAGTTATCTAAGTTAATTTAAAGTTGTTTGTGC
2777 CTTTTTATTTTTGTTTTTAATGCTTTGATATTTCAATGTTAGCCTCAATTTCTGAACACC
2837 ATAGGTAGAATGTAAAGCTTGTCTGATCGTTCAAAGCATGAAATGGATACTTATATGGAA

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Figure ²1. In situ hybridization detection of CTGF expression in humerus of newborn mouse.



Figure 4. Effect of CTGF on the differentiation of C3H10T1/2 cells.



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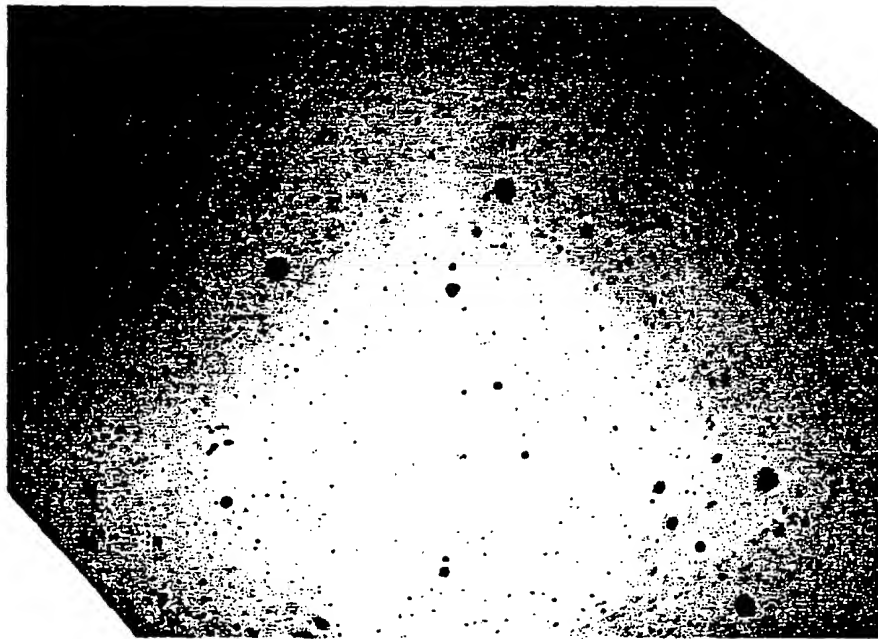


FIG 7A

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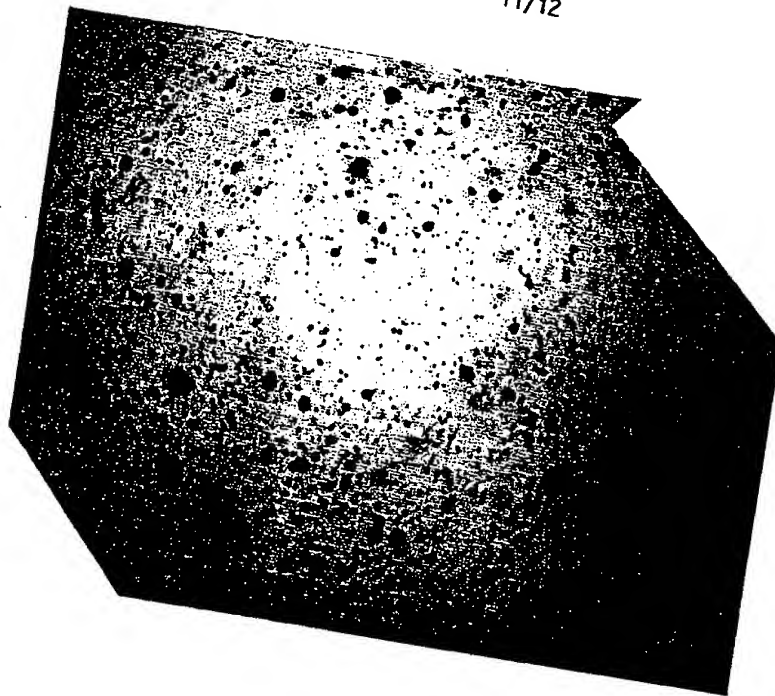


FIG 7C

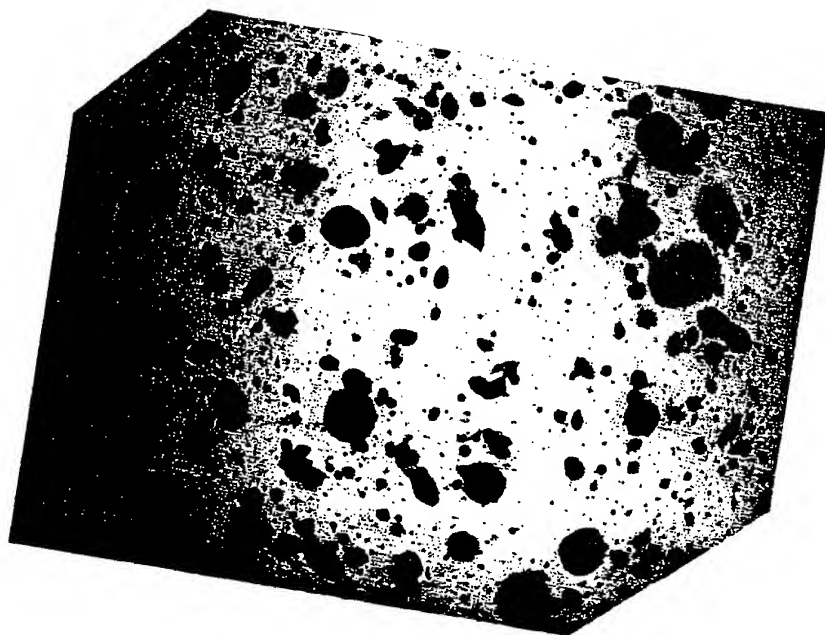


FIG 7D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/08210

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/18, 38/39; C07K 14/495, 14/475, 14/78

US CL : 424/198.1, 423, 484, 492; 530/350, 356

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1, 423, 484, 492; 530/350, 356

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

search terms: connective tissue growth factor, TGF-beta, collagen, wound, bone, cartilage

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,408,040 (GROTENDORST ET AL.) 18 April 1995, see entire document, especially column 2, column 5.	1, 8-10, 16-18 ----- 2-7, 11-15, 19
Y	US, A, 5,149,691 (RUTHERFORD) 22 September 1992, see the claims.	2-7, 11-15, 19
Y	US, A, 5,356,630 (LAURENCIN ET AL.) 18 October 1994, see claim 1.	2-7, 11-15, 19
Y	US, A, 5,399,361 (SONG ET AL.) 21 March 1995, see column 2.	2-7, 11-15, 19

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 JULY 1996

Date of mailing of the international search report

22 JUL 1996

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